

EXHIBIT BO

D 8-1

Experimental report

Under my supervision the following experiments have been performed in the discovery research laboratories in Tsukuba, Nippon Shinyaku Co. as described in more detail below:

Materials and Methods

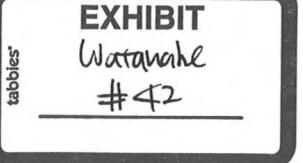
Antisense Oligonucleotides

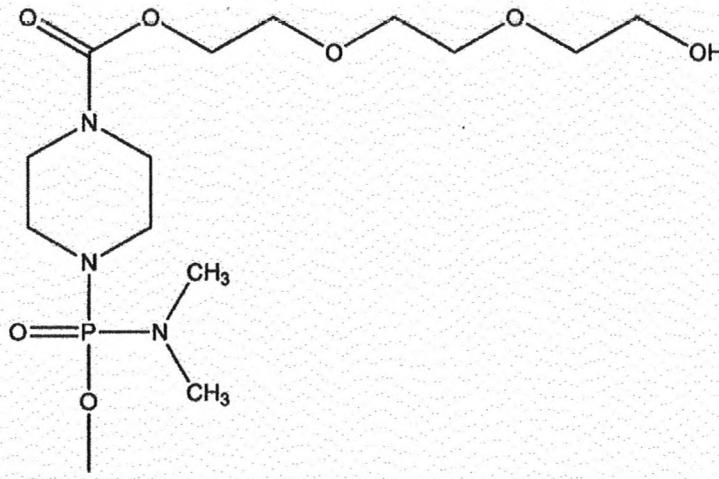
PMOs of SEQ ID NOs: 1–4 shown in Table 1 below, which cause skipping of the 53rd exon in the human dystrophin gene, were synthesized as described in patent no. EP 2612917. Each synthesized PMO was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.).

Table 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NOs:
45–62	5'-CTGTTGCCCTCCGGTTCTG-3'	SEQ ID NO: 1
49–69	5'-CATTCAACTGTTGCCTCCGGT-3'	SEQ ID NO: 2
50–69	5'-CATTCAACTGTTGCCTCCGG-3'	SEQ ID NO: 3
39–69	5'-CATTCAACTGTTGCCTCCGGTTCTGAAGGTG-3'	SEQ ID NO: 4

Antisense oligomers with the above sequences, wherein the 5' end is the group of chemical formula (1) below, were prepared.





Formula (1)

SEQ ID NO: 1 ESI-TOF-MS Calculated: 6239.18 Measured: 6238.91

SEQ ID NO: 2 ESI-TOF-MS Calculated: 7216.53 Measured: 7216.72

SEQ ID NO: 3 ESI-TOF-MS Calculated: 6886.42 Measured: 6886.83

SEQ ID NO: 4 ESI-TOF-MS Calculated: 10620.69 Measured: 10620.96

The localization of the oligonucleotides in the relevant area is shown in the enclosed annex A

Cells

RD cells were obtained from the Health Science Research Resources Bank and cultured under 5% CO₂ at 37°C in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum.

Transfection of PMO into cells

PMOs were dissolved in distilled water and transfected into RD cells using Amaxa cell line Nucleofector kit L and a Nucleofector II device (Lonza, Basel, Switzerland) with program T-030.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from RD cells using a QIAshredder spin column (Qiagen, Valencia, CA, USA) and an RNeasy mini kit (Qiagen). RNA concentrations were determined by the absorbance at 260 nm by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed with 400 ng of the extracted total RNA using a Qiagen OneStep RT-PCR Kit (Qiagen). The forward primer was 5'-CTG AGT GGA AGG CGG

TAA AC-3' and the reverse primer was 5'-GAA GTT TCA GGG CCA AGT CA-3'. RT-PCR was performed using an RTC-100 thermocycler (MJ Research, Watertown, MA, USA). The RT-PCR program was as follows: reverse transcription at 50°C for 30 min and heat denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR reaction products were analyzed using a 2100 Bioanalyzer (Agilent, Waldbronn, Germany). The % skipping efficiency was determined from the expression $\frac{[\text{PCR reaction products without exon 53}]}{[\text{PCR reaction products without exon 53}] + [\text{PCR reaction products with exon 53}]} \times 100$.

Results

The following results were measured:

% Skipping efficiency

Concentration (μM)		10	30
SEQ ID NO: 1	45-62	5.9 ± 1.8	15.1 ± 10.9
SEQ ID NO: 2	49-69	2.0 ± 0.5	5.5 ± 2.0
SEQ ID NO: 3	50-69	3.0 ± 3.4	7.1 ± 3.0
SEQ ID NO: 4	39-69	18.3 ± 3.6	24.7 ± 4.4

mean ± S.D.

This means that SEQ ID NO: 1 and SEQ ID NO: 4 induced exon 53 skipping in vitro more strongly than SEQ ID NO: 2 or SEQ ID NO: 3 did. Since the activity of the various oligonucleotides differs substantially it is evident that the invention cannot be worked successfully over the whole scope of the claim.

I, Mr. Naoki Watanabe declare that the experiments have been performed under my supervision
and that I have measured the results as presented in this experimental report.

Naoki Watanabe

Mr. Naoki Watanabe

Address 3-14-1, Sakura, Tsukuba, Ibaraki Japan

Date September 26, 2017

Annex A

	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
mRNA	5'	G	G	A	U	G	A	A	G	U	A	C	A	A	G	A	A	C	A	C	C	U	U	C	A	G	A	C	C	G	G	C	A	A	G	U	U	G	A	A	U	G	-3'				
23-47																																															
39-69	SEQ ID NO: 4																																														
45-62	SEQ ID NO: 1																																														
48-69	SEQ ID NO: 5																																														
47-68	SEQ ID NO: 6																																														
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47-67	SEQ ID NO: 8																																														
49-68	SEQ ID NO: 9																																														
48-67	SEQ ID NO: 10																																														
antisense	3'	C	C	U	A	I	C	U	U	C	A	U	G	U	U	C	I	U	U	G	U	G	G	A	A	G	U	I	C	I	U	U	G	I	G	I	C	I	C	I	U	I	A	I	C	-5'	

Annex A

EXHIBIT BP

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EXHIBIT BQ

D13

Experimental report

The purpose of the study reported herein is to demonstrate that claimed invention in the European Patent EP2206781 includes antisense oligonucleotides (AON) that do not have sufficient skipping activity against exon 53 of the human dystrophin gene.

Under my supervision the following experiments have been performed in the discovery research laboratories in Tsukuba, Nippon Shinyaku Co. as described in more detail below:

Materials and Methods

Antisense Oligonucleotides

PMOs of SEQ ID NOs: 1, 4 and 5 to 10 shown in Table 1 below, which cause skipping of exon 53 in the human dystrophin gene, were synthesized in accordance with the method set forth in European Patent EP 2612917. Each synthesized PMO was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.).

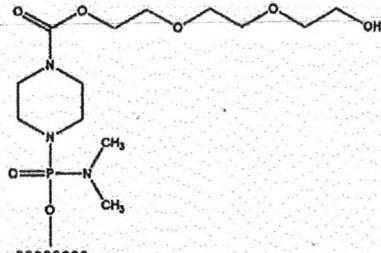
Table 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NOs.
45-62	5'-CTGTTGCCTCCGGTCTG-3'	SEQ ID NO: 1
39-69	5'-CATTCAACTGTTGCCTCCGGTTCTGAAGGTG-3'	SEQ ID NO: 4
48-69	5'-CATTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 5
47-68	5'-ATTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 6
48-68	5'-ATTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 7
47-67	5'-TTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 8
49-68	5'-ATTCAACTGTTGCCTCCGGT-3'	SEQ ID NO: 9
48-67	5'-TTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 10

Antisense oligomers with the above sequences, wherein the 5' end is the group of chemical formula (1) below, were prepared.

EXHIBIT
Watanabe
44

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Formula (1)

SEQ ID NO: 1 ESI-TOF-MS Calculated: 6239.18 Measured: 6238.91
SEQ ID NO: 4 ESI-TOF-MS Calculated: 10620.69 Measured: 10620.96
SEQ ID NO: 5 ESI-TOF-MS Calculated: 7546.64 Measured: 7546.52
SEQ ID NO: 6 ESI-TOF-MS Calculated: 7546.64 Measured: 7546.98
SEQ ID NO: 7 ESI-TOF-MS Calculated: 7231.53 Measured: 7231.81
SEQ ID NO: 8 ESI-TOF-MS Calculated: 7207.52 Measured: 7207.04
SEQ ID NO: 9 ESI-TOF-MS Calculated: 6901.42 Measured: 6901.54
SEQ ID NO: 10 ESI-TOF-MS Calculated: 6892.41 Measured: 6892.83

The localization of the oligonucleotides in the relevant area is shown in the enclosed annex A

Cells

RD cells were obtained from the Health Science Research Resources Bank and cultured under 5% CO₂ at 37°C in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum.

Transfection of PMO into cells

The PMOs were dissolved in distilled water and transfected into RD cells using Amaxa cell line Nucleofector kit L and a Nucleofector II device (Lonza, Basel, Switzerland) with program T-030.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from RD cells using a QIAshredder spin column (Qiagen, Valencia, CA, USA) and an RNeasy mini kit (Qiagen). RNA concentrations were determined by the absorbance at 260 nm by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed with 400 ng of the extracted total RNA using a Qiagen OneStep RT-PCR Kit (Qiagen). The forward primer was 5'-CTG AGT GGA AGG CGG TAA AC-3' and the reverse primer was 5'-GAA GTT TCA GGG CCA AGT CA-3'. RT-PCR was performed using an RTC-100 thermocycler (MJ Research, Watertown, MA, USA). The RT-PCR

program was as follows: reverse transcription at 50°C for 30 min and heat denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR reaction products were analyzed using a 2100 Bioanalyzer (Agilent, Waldbronn, Germany). The skipping efficiency was determined from the expression $[PCR \text{ reaction products without exon 53}] \times 100 / ([PCR \text{ reaction products without exon 53}] + [PCR \text{ reaction products with exon 53}])$.

Results

The following results were measured:

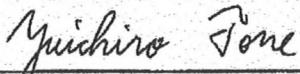
Skipping efficiency (%)

PMOs		10(μM)	30(μM)
SEQ ID NO: 1	45-62	6.0 ± 3.2	14.3 ± 4.1
SEQ ID NO: 4	39-69	15.9 ± 1.2	21.5 ± 2.7
SEQ ID NO: 5	48-69	2.8 ± 1.7	2.0 ± 1.9
SEQ ID NO: 6	47-68	1.1 ± 1.9	2.5 ± 1.3
SEQ ID NO: 7	48-68	2.3 ± 2.0	2.2 ± 1.0
SEQ ID NO: 8	47-67	1.6 ± 0.8	4.6 ± 1.8
SEQ ID NO: 9	49-68	2.8 ± 0.5	2.0 ± 1.8
SEQ ID NO: 10	48-67	2.2 ± 1.4	2.6 ± 2.6

mean \pm S.D.

The results clearly show that apart from SEQ ID NO: 1 and SEQ ID NO:4 having relatively high skipping activity for exon 53 in vitro, none of SEQ ID NOs: 5–10 have sufficient skipping activity. Since the activity of the tested PMOs differs substantially, it is evident that the claimed invention cannot work successfully over the whole scope of the claim.

I, Mr. Yuichiro Tone declare that the experiments have been performed under my supervision and that I have measured the results as presented in this experimental report.



Mr. Yuichiro Tone

Address 3-14-1, Sakura, Tsukuba, Ibaraki, Japan
Date September 26, 2017

Annex A

	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69			
mRNA	5'	G	G	A	U	G	A	A	G	U	A	C	A	G	A	A	C	A	C	C	U	U	C	A	G	A	A	C	C	G	G	C	A	A	C	A	G	U	U	G	A	A	U	G	-3'					
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Annex A

EXHIBIT BR

REDACTED
IN ITS
ENTIRETY

EXHIBIT BS

REDACTED
IN ITS
ENTIRETY

EXHIBIT BT

Experimental Report

(1) Methods

The following antisense oligonucleotides were synthesized in the same manner as described in the present specification (patent publication no. EP3018211) by an automated synthesizer (AKTA oligopilot plus 10 (GE Healthcare)).

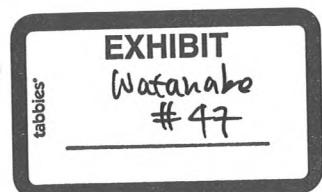
H53_36-60: 5'- GTGCCTCCGGTTCTGAAGGTGTT -3'; corresponding to SEQ ID NO: 57 of the present application, and complementary to the 36th to the 60th nucleotides from the 5' end of the human dystrophin gene's 53rd exon; and

H53_33-62: 5'- CTGTTGCCTCCGGTTCTGAAGGTGTTCTTG-3'; corresponding to H53A30/2 of D1, and complementary to the 33rd to the 62nd nucleotides from the 5'- end of the human dystrophin gene's 53rd exon.

H53_36-56: 5'- CCTCCGGTTCTGAAGGTGTT -3'; corresponding to SEQ ID NO: 35 of the present application, and complementary to the 36th to the 56th nucleotides from the 5' end of the human dystrophin gene's 53rd exon; and

The skipping efficiencies of H53_36-60 and H53_33-62 were measured in separate *in vitro* experiments but both experiments included H53_36-56 as control. Briefly, 10 µM of oligonucleotides H53_36-60, H53_33-62 and H53_36-56 were transfected with 3.5×10^5 of RD cells with an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza). The Program T-030 was used in the transfection. The transfected cells were cultured for three days in 2 mL of Eagle's minimal essential medium (EMEM)(Sigma) containing 10% fetal bovine serum (FBS) (Invitrogen) under 37°C and 5% CO₂. After washing the cells with PBS (Nissui), total RNA was extracted from RD cells using a QIAshredder spin column (Qiagen, Valencia, CA, USA) and an RNeasy mini kit (Qiagen) with a protocol suggested by the manufacturer. RT-PCR was performed with 400 ng of the extracted total RNA, using a QIAGEN OneStep RT-PCR Kit (Qiagen) and primer pairs having the nucleotide sequences of 5'- CTGAGTGGAAAGCGGTAAAC -3' and 5'- GAAGTTTCAGGGCCAAGTCA -3'. One microliter of the RT-PCR product was analyzed with a Bioanalyzer (Agilent Technologies, Inc.).

The exon 53 skipping efficiency was calculated as below:



$$\text{Skipping efficiency}(\%) = \frac{A}{(A+B)} \times 100$$

wherein “A” refers to the polynucleotide level of the band with exon 53 skipping, and “B” refers to the polynucleotide level of the band without exon 53 skipping. Experiment was repeated three times for each oligonucleotide.

(2) Results

Experiment 1

Sequences	Concentration (μM)	Repeat number	Skipping efficiency(%)	
			Mean	Standard deviation
H53_36-60	10	3	45.5	10.9
H53_36-56	10	3	68.0	1.9

Experiment 2

Sequences	Concentration (μM)	Repeat number	Skipping efficiency(%)	
			Mean	Standard deviation
H53_33-62	10	3	32.7	4.5
H53_36-56	10	3	76.2	8.0

The data show that H53_36-60 showed higher skipping efficiency in experiment 1 than H53_33-62 did in experiment 2, although control sequence of H53_36-56 showed lower skipping efficiency in test 1 than in test 2. Thus, the presently claimed oligomer H53_36-60 has superior skipping activity over H53A30/2 of D1.

Declaration of Toshihiro Ueda concerning EP 3 018 211 B1

I, Toshihiro Ueda, am a Principal of Discovery Research Laboratories of Nippon Shinyaku Co., Ltd. located in Tsukuba and an expert in the field of antisense oligomers as therapeutic agents for Duchenne Muscular Dystrophy. I am the person that supervised and was responsible for the experiments in the "Experimental Report" that was filed during the examination phase of EP 3 018 211 at the European Patent Office on behalf of Nippon Shinyaku Co., Ltd. and the National Center of Neurology and Psychiatry by HGF with their letter dated 16 March 2018.

I herewith confirm that all of the antisense oligomers used in the above mentioned "Experimental Report" submitted during examination, i.e., the antisense oligomers "H53_36-60", "H53_33-62" and "H53_36-56", contained an -OH group at their 5'-end.

September 29, 2020, Tsukuba, Japan Toshihiro Ueda
Date, Place Name